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FOR: METHOD OF CONSTRUCTING HOST AND METHOD OF PRODUCING HETEROLOGOUS PROTEIN

DECLARATION

COMMISSIONER FOR PATENTS

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SIR:

Now comes TOMOKO CHIBA who deposes and says:

That my name is TOMOKO CHIBA;

That my address is 3-26 Naka-aoki 1-chome, Kawaguchi-shi, Saitama-ken, Japan;

That I know well both the English and Japanese languages;

That the attached English language translation is true and correct translation of Japanese Patent Application No. JP2001-160128 filed on May 29, 2001 to the best of my knowledge and belief;

I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

FURTHER DEPONENT SAITH NOT.

September 24, 2008
Date

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Applicant(s):	Asahi Glass Company, Limited

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【TYPE OF DOCUMENT】 SPECIFICATION

【TITLE OF THE INVENTION】

METHOD OF CONSTRUCTING HOST AND METHOD OF PRODUCING HETEROLOGOUS PROTEIN

【SCOPE OF THE CLAIM(S)】

【Claim 1】

A method of constructing a eukaryotic host microorganism for production of a heterologous protein encoded by a transgenically introduced gene, which is characterized by deleting or inactivating part or all of the genome of a eukaryotic host microorganism unnecessary or detrimental to production of the heterologous protein by a transformant of the host in culture for the purpose of improving productivity of the heterologous protein.

【Claim 2】

The method according to Claim 1, wherein the part of the genome unnecessary or detrimental to production of the heterologous protein by the transformant in culture is genes associated with energy metabolism, proteases, meiosis, transcription, cell growth and division and DNA synthesis, protein synthesis, membrane transport, cell structure maintenance, signal transduction or ion homeostasis in the eukaryotic host microorganism.

【Claim 3】

The method according to Claim 1 or 2, wherein the eukaryotic host microorganism is *Schizosaccharomyces*

pombe.

【Claim 4】

The method according to Claim 3, wherein the part of the genome of *Schizosaccharomyces pombe* unnecessary or detrimental to production of the heterologous protein by the transformant in culture is a gene selected from the genes associated with energy metabolism and the genes associated with proteases.

【Claim 5】

A eukaryotic host microorganism for production of a heterologous protein encoded by a transgenically introduced gene, which is constructed by the method according to Claim 1 or 2.

【Claim 6】

The host according to Claim 5, wherein the eukaryotic microorganism is *Schizosaccharomyces pombe*.

【Claim 7】

A transformant obtained by introducing the structural gene encoding a heterologous protein into a eukaryotic host microorganism in which part or all of the genome of the eukaryotic host microorganism unnecessary or detrimental to production of the heterologous protein by the transformant in culture has been deleted or inactivated for the purpose of improving productivity of the heterologous protein.

【Claim 8】

The transformant according to Claim 7, wherein the

part of the genome unnecessary or detrimental to production of the heterologous protein by the transformant in culture is genes associated with the energy metabolism, proteases, meiosis, transcription, cell growth and division and DNA synthesis, protein synthesis, membrane transport, cell structure maintenance, signal transduction or ion homeostasis in the eukaryotic host microorganism.

【Claim 9】

The transformant according to Claim 7 or 8, wherein the eukaryotic microorganism is *Schizosaccharomyces pombe*.

【Claim 10】

A method of producing a heterologous protein, comprising causing a transformant of a eukaryotic host microorganism having a gene encoding a heterologous protein extrinsic to the host and collecting the heterologous protein, wherein the productivity of the heterologous protein is improved by deleting or inactivating part or all of the genome of the eukaryotic host microorganism which is unnecessary or detrimental to production of the heterologous protein by the transformant in culture.

【Claim 11】

The method according to Claim 10, wherein the part of the genome unnecessary or detrimental to production of the heterologous protein by the transformant in culture is genes associated with the energy metabolism, proteases,

meiosis, transcription, cell growth and division and DNA synthesis, protein synthesis, membrane transport, cell structure maintenance, signal transduction or ion homeostasis in the eukaryotic host microorganism.

【Claim 12】

The method according to Claim 10 or 11, wherein the eukaryotic microorganism is *Schizosaccharomyces pombe*.

【Claim 13】

The method according to Claim 12, wherein the part of the genome unnecessary or detrimental to production of the heterologous protein by the transformant in culture is a pyruvate decarboxylase gene or a serine protease gene.

【DETAILED DESCRIPTION OF THE INVENTION】

【Technical Field to which the Invention Belongs】

The present invention relates to a eukaryotic host microorganism in which part of the genome of the eukaryotic microorganism is modified for the purpose of improving the productivity of a heterologous protein by a transformant of the eukaryotic host microorganism, a method of constructing the host, a trasnformant of the host and a method of producing a protein using the transformant. The eukaryotic microorganism is preferably the fission yeast, *Schizosaccharomyces pombe* (hereinafter referred to as *S. pombe*).

【Prior Art】

Recombinant DNA technology is used for production of

heterologous proteins in various host microorganisms and animals including *Escherichia coli* (hereinafter referred to as *E. coli*). The target products are various biogenous proteins (herein, inclusive of polypeptides), and many of them have already been produced industrially for medical and other uses so far.

Among various hosts developed for production of heterologous proteins, yeasts seem favorable for expression of animal and plant proteins because of their eukaryotic similarity in the transcription and translation systems to animals and plants, and the baker's yeast (*Saccharomyces cerevisiae*) is a widely used host. Among yeasts, *S. pombe* is known to be close to animal cells in nature as is evident from the fact that it grows by fission not by budding as a result of the different evolution process it has followed since it diverged from other yeasts at early stages. Therefore, the use of *S. pombe* as the host for expression of heterologous proteins is expected to provide a gene product closer to its natural form in animal cells.

Though studies of gene expression in *S. pombe* are delayed, the recent discovery of potent promoters functional in *S. pombe* has accelerated the development of expression systems using *S. pombe* as the host, and various improvements have been added to expression vectors to develop more stable and efficient expression systems (Japanese Patent No. 2776085, JP-A-07-163373, JP-

A-10-215867, JP-A-10-234375, JP-A-11-192094, JP-A-2000-136199, JP-A-2000-262284). As a result, expression systems using *S. pombe* as the host show high production efficiency now.

【Problems that the Invention is to Solve】

Production systems for heterologous proteins using eukaryotic microorganisms such as yeasts can be realized easily by conventional microbiological techniques and recombinant DNA technology with high productivity. Large cultures are already available and are acceleratingly used for actual production. Even after the scale is enlarged for actual production, cells retain the high production efficiency per cell obtained in the laboratory.

However, considering that cost reduction is often demanded in actual production, it is necessary to improve the production efficiency of heterologous proteins through improvement in cell growth efficiency, suppression of degradation of the heterologous protein of interest, more efficient eukaryotic modifications in the microorganisms or more efficient utilization of the nutrition sources. For example, increase in the conversion of the carbon sources added to the medium for culture growth into the heterologous protein of interest is expected to drastically improve cell growth efficiency and therefore production efficiency of the heterologous protein, because efficient utilization of the carbon sources in the medium for production of the heterologous

protein of interest seems to be sacrificed for their consumption by metabolic systems unnecessary for cell growth or production of the heterologous protein of interest (such as the ethanol fermentation system for production of ethanol).

【Means of Solving the Problems】

Under the above-mentioned circumstance, the present inventors studied from the above-mentioned aspects, and, as a result, found that the deletion or inactivation of part or all of the genome of the host unnecessary or detrimental to production of the heterologous protein by its transformant improves the production efficiency of the heterologous protein. The present invention aims at improvement in the production efficiency of a heterologous protein, relates to a method of constructing a eukaryotic host microorganism, a host constructed by the construction method, a transformant of the host obtained by introducing a gene encoding a heterologous protein into the host and a method of producing a heterologous protein using the transformant, and provides:

(1) a method of constructing a eukaryotic host microorganism for production of a heterologous protein encoded by a transgenically introduced gene, which is characterized by deleting or inactivating part or all of the genome of a eukaryotic host microorganism unnecessary or detrimental to production of the heterologous protein

by a transformant of the host in culture for the purpose of improving productivity of the heterologous protein;

(2) a eukaryotic host microorganism for production of a heterologous protein encoded by a transgenically introduced gene, which is constructed by the construction method;

(3) a transformant obtained by introducing the structural gene encoding a heterologous protein into a eukaryotic host microorganism in which part or all of the genome of the eukaryotic host microorganism unnecessary or detrimental to production of the heterologous protein by the transformant in culture has been deleted or inactivated for the purpose of improving productivity of the heterologous protein; and

(4) a method of producing a heterologous protein, comprising causing a transformant of a eukaryotic host microorganism having a gene encoding a heterologous protein extrinsic to the host and collecting the heterologous protein, wherein the productivity of the heterologous protein is improved by deleting or inactivating part or all of the genome of the eukaryotic host microorganism which is unnecessary or detrimental to production of the heterologous protein by the transformant in culture.

The part of the genome unnecessary or detrimental to production of the heterologous protein by the transformant in culture is preferably genes associated

with energy metabolism, proteases, meiosis, transcription, cell growth and division and DNA synthesis, protein synthesis, membrane transport, cell structure maintenance, signal transduction or ion homeostasis.

The eukaryotic microorganism is preferably a yeast, especially *S. pombe*. The part of the genome unnecessary or detrimental to production of the heterologous protein by *S. pombe* is a gene selected from the genes associated with a pyruvate decarboxylase gene and a serine protease gene.

【Mode of Carrying out the Invention】

In the present invention, the eukaryotic microorganism is preferably a fungus, especially a unicellular fungus (i.e., a yeast). As the yeast, a yeast of the *Saccharomyces* genus such as the baker's yeast, a yeast of the *Shizosaccharomyces* genus such as *S. pombe* or a yeast of the *Pichia* genus is preferable. Eukaryotic microorganisms of the *Aspergillus* genus, the *Rhizopus* genus or the *Penicillium* genus and other eukaryotic microorganism may also be mentioned. The eukaryotic microorganism particularly preferred in the present invention is a yeast of the *Schizosaccharomyces* genus, especially *S. pombe*. Hereinafter, hosts mean those eukaryotic microorganisms, unless otherwise noted.

It is common in recent years to transgenically introduce the gene (hereinafter referred to as a heterologous gene) encoding a protein extrinsic to a host

(i.e., a heterologous protein) to the host and causing the host having the introduced heterologous gene (i.e., a trasnformant) to produce the heterologous protein and collecting the heterologous protein. While the culture of the transformant is producing the heterologous protein, part of the genome is unnecessary or detrimental to production of the heterologous protein by the transformant in culture. The part of the genome may be a gene or a nongenomic part, preferably a genomic part of the genome. Deletion or inactivation of the gene improves the production efficiency of the heterologous protein by the transformant. It is believed that a lot of such unnecessary or detrimental genes exist in a genome. Deletion or inactivation of part of these genes sufficiently meets the purpose of the present invention.

The part of the genome unnecessary or detrimental to production of the heterologous protein by the transformant may be genes essential for the wild type host to survive or grow, because such essential genes are not always necessary to a transformant culture. For example, the genes essential for conversion of carbon sources to nutrients are no longer necessary if the nutrients are added to the culture environment (culture medium). Meanwhile, in the case of yeasts which can grow not by meiosis but by budding or fission, genes associated with meiosis are not always necessary for the transformant culture. The existence of such unnecessary

genes can be a burden to growth of the transformant or production of the heterologous protein. Therefore, deletion or inactivation of such genes lightens the burden and improves the production efficiency of the heterologous protein.

On the other hand, genes associated with proteases tend to inhibit the production of the heterologous protein. Because the heterologous protein produced is fundamentally unnecessary to the host, the transformant tends to degrade the produced heterologous protein by proteases. Since degradation of the heterologous protein is considered as a factor of reduction in the production efficiency of the heterologous protein, deletion or inactivation of the genes associated with production of such proteases improves the production efficiency of the heterologous protein.

Such genes unnecessary or detrimental to production of the heterologous protein as described above are preferably genes associated with energy metabolism, proteases, meiosis, transcription, cell growth and division and DNA synthesis, protein synthesis, membrane transport, cell structure maintenance, signal transduction or ion homeostasis. Particularly preferred are genes selected from the genes associated with energy metabolism and the genes associated with proteases.

The preferable gene in the genes associated with energy metabolism is a gene associated with ethanol

fermentation. A typical example of the genes associated with ethanol fermentation is the gene encoding pyruvate decarboxylase (the pyruvate decarboxylase gene). Deletion or inactivation of the pyruvate decarboxylase gene is considered to make the culture of the transformant distribute more energy to synthetases instead of ethanol fermentation and thereby improves the production efficiency of the heterologous protein.

The genes associated with proteases include genes encoding endopeptidases such as serine proteases and exopeptidases such as aminopeptidases. Particularly preferred are genes encoding serine proteases (serine protease genes). Deletion or inactivation of these genes associated with proteases is considered to improve the production efficiency of the heterologous protein.

Part of the genome of the host can be deleted or inactivated by known methods. One or more parts of the genome may be deleted or inactivated. When the part to be deleted or inactivated is gene(s), deletion or inactivation may be effected on a single gene or two or more individual genes. Part of the gene to be deleted or inactivated may be a structural sequence or a regulatory sequence.

Deletion of a gene may be deletion of the entire gene or deletion of part of the gene for inactivation of the gene. Inactivation of a gene means not only deletion of part of the gene but also modification of the gene

without deletion. A gene may be inactivated by inserting another gene or DNA into a certain sequence in the gene as the inactivation target. In any case, the target gene is inactivated so as to encode an inactive protein or so as to be unable to be transcribed or translated.

Though there is no restriction on the heterologous protein, it is preferably a protein which is produced by multicellular organisms such as animals and plants, especially a protein produced by a mammal (inclusive of human). Such a protein is rarely obtained with high activity by a prokaryotic host microorganism such as *E. coli* and is obtained with low production efficiency by using an animal cell line such as CHO as the host. The use of the transgenic eukaryotic host microorganism of the present invention is considered to solve these problems.

【Examples】

Now, the present invention will be described in further detail in reference to specific Examples.

【Example 1】

Improvement in the production efficiency of *Aequorea victria* green fluorescent protein by inactivation of the pyruvate decarboxylase gene pdc1

A 1.8-kb fragment from the orotidine phosphate decarboxylase gene of *S. pombe* was inserted in the 1785-bp ORF (the protein-coding region) of the pyruvate decarboxylase gene pdc1 (SPAC1F8.07c) of the fission yeast

S. pombe to obtain a pdc1-disrupted vector. A green fluorescent protein-producing uracil-requiring auxotroph (obtained by inactivating the orotidine phosphate decarboxylase activity of the yeast strain used in the octuplicated integrative production system disclosed in JP-A-2000-262284 through gene disruption) was transformed with the vector. A uracil-unrequiring strain capable of forming colonies on the minimum medium was collected. Analysis of the genomic DNA by PCR amplification confirmed disruption of the pyruvate decarboxylase gene.

The transformant was grown and tested for green fluorescent protein production in YPD liquid medium (1% yeast extract (DIFCO), 2% Bacto-Peptone (DIFCO), 2% glucose (Wako Pure Chemical Industries, Ltd.)) in test tube-shaped culture vessels. The production per cell was higher than in the original strain, according to fluorometry using a microplate reader (CORONA, MTP-32+MTPF2) at an excitation wavelength of 490 nm and an emission wavelength 530 nm.

【Example 2】

Improvement in the production efficiency of *Aequorea victria* green fluorescent protein by inactivation of the serine protease gene isp6

A 1.8-kb fragment from the orotidine phosphate decarboxylase gene of *S. pombe* was inserted in the 1404 bp ORF of a serine protease gene isp6 (SPAC4A8.04) of the fission yeast *S. pombe* to obtain an isp6-disrupted vector.

The same green fluorescent protein-producing uracil-requiring auxotroph as in Example 1 was transformed with the vector. A uracil-unrequiring strain capable of forming colonies on the minimum medium was collected. Analysis of the genomic DNA by PCR amplification confirmed disruption of the serine protease gene.

The transformant was grown in the same manner as in Example 1 and tested for green fluorescent protein production. The production per cell was higher than in the original strain, according to fluorometry using a microplate reader (CORONA, MTP-32+MTPF2) at an excitation wavelength of 490 nm and an emission wavelength 530 nm.

【Effects of the Invention】

Inactivation of the pyruvate decarboxylase gene or a serine protease gene in the fission yeast *S. pombe* improved production efficiency of a heterologous protein in a transformant of the fission yeast host S. pombe. Thus, in a protein production system using a transformant of a eukaryotic host microorganism having a transgenically introduced gene encoding a heterologous protein, deletion or inactivation of part of a genome unnecessary or detrimental to production of the heterologous protein by the transformant in culture improves production efficiency of the heterologous protein.

【TYPE OF DOCUMENT】 ABSTRACT

【SUMMARY】

【OBJECT】

In a method of constructing a eukaryotic host microorganism for production of a heterologous protein encoded by a trangenically introduced gene, production efficiency of the heterologous protein by the transformant obtained by introducing the gene encoding the heterologous protein into the host is improved.

【MEANS OF SOLVING PROBLEMS】

Part or all of the genome unnecessary or detrimental to production of the heterologous protein by the transformant in culture is deleted or inactivated.

【SELECTED FIGURE】 No Selected Figure